REMARKS

The present application relates to inbred maize plant and seed PH581. Claims 1-30 are pending in the present application. Claims 19-22 have been amended and claim 30 has been canceled. No new matter has been added by way of amendment. Applicants respectfully request consideration of the claims in view of the following remarks.

Detailed Action

Applicants acknowledge that because this application is eligible for continued examination under 37 C.F.R. § 1.114, and the fee set forth in 37 C.F.R. § 1.17(e) has been timely paid, the finality of the previous Office Action has been withdrawn pursuant to 37 C.F.R. § 1.114. Applicants further acknowledge that Applicants' submission filed on October 18, 2005 has been entered.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 19-24 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regards as the invention.

Regarding claims 19 and 23-24, the Examiner states the claims "are indefinite in their recitation of 'plant of claim 11 . . . further defined as . . . comprising a single locus conversion [or a gene or a transgene]'. It is confusing to characterize the plant of claim 11, which has a finite and particularly defined genome comprising a particular allele at every individual locus, as simultaneously comprising additional transgene or single locus conversions." The Examiner additionally states that it is "confusing to characterize the male fertile plant of claim 11 as simultaneously male sterile." *See* Office Action, p. 2

Applicants respectfully traverse this rejection. The Specification teaches that a gene conferring male sterility can be introduced into a maize plant using techniques well known in the art. (*See* Specification, p. 2, l. 25-p. 3, l. 6; p. 20, ll. 16-34) One skilled in the art would thus recognize that Applicants have adequately described claims 23 and 24.

Further, claims 19 and 23-24 depend from claim 11, which is adequately described and enabled. Claim 11 has been deemed allowable by the Examiner. Although not acceding to the Examiner's rejection, in an effort to expedite prosecution Applicants have amended claim 19 to

read "single gene conversion", further defining the claims. Applicants respectfully submit that one skilled in the art would thus recognize that Applicants have adequately defined claims 19 and 23-24.

Regarding claim 22, the Examiner states the claim is "indefinite in its recitation of 'yield enhancement' and 'improved nutritional quality' as these are relative terms for which no comparative standard is provided." *See* Office Action, p. 3.

Applicants respectfully traverse this rejection. "Yield Advantage" is defined in the specification as "the yield advantage of variety #1 over variety #2". (Specification, p. 16, ll. 10-11). Therefore yield enhancement would be the improvement of the trait yield over another variety. Applicants assert that genes which increase yield by increasing the plants resistance to disease, herbicides, or insects are within the scope of the claims as presented.

Similarly, "improved nutritional quality" would represent an improvement in the nutritional quality versus another variety as described on page 22 of the specification. Further, single genes that affect nutritional quality are known in the art. Specifically genes for modified fatty acids, decreased phytate content and modified carbohydrate compositions are disclosed in the specification. (Specification, p. 33, l. 17-p. 34, l. 5). Applicants respectfully submit that one skilled in the art would thus recognize that claim 22 is adequately defined.

In light of the above amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

Rejections Under 35 U.S.C. § 112, First Paragraph

A. Written description regarding Claim 30

Claim 30 stands rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. The claim(s) contains subject matter, which was not described in the specification in such a way as reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states that the "claim is drawn to a plant breeding method comprising obtaining a molecular marker profile of the exemplified inbred, inducing doubled haploidy of F1 hybrid seed produced from the inbred, and then selecting progeny that retain the molecular marker profile of the exemplified inbred. However, no basis in the specification was provided for these terms". *See* Office Action, p. 3

In an effort to expedite prosecution, Applicants have canceled claim 30, thereby alleviating this rejection.

B. Written description regarding Claims 1-10, 13-16 and 18-30

Claims 1-10, 13-16 and 18-30 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The Examiner asserts that the claims(s) contains subject matter, which was not described in the specification in such a way as reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner states the rejection is repeated for claims 1-10 and applied to new claims 13-16 and 18 for the reasons of record set forth in the Office Action of December 17, 2004. The Examiner further states that claims 25-30 are included "because they are drawn to methods of using uncharacterized descendants of the exemplified inbred in a multitude of outcrossing steps to uncharacterized breeding partners." *See* Office Action, pp. 3-4

Applicants respectfully traverse this rejection. Applicants reiterate that the written description requirement of § 112, first paragraph has been fulfilled by depositing seeds of PH581 in a public depository and by referencing the deposit in the specification (p. 50, ll. 2-28). *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 965, 63 U.S.P.Q.2d 1609, 1613 (Fed. Cir. 2002). (stating that the written description requirement of § 112, ¶ 1 may be fulfilled by depositing material in a public depository, where the deposited material is not accessible in writing, and where reference to the deposit is made in the specification). This deposit not only describes inbred maize line PH581 but also the hybrid maize plants, plant parts, and seeds grown of claims 1-10, 13-16 and 18-30. In a prior case before the Board of Patent Appeals and Interferences, the Board determined that where an inbred maize plant had been deemed allowable, claims to the F1 hybrid seed and plants resulting from a cross between the allowable inbred maize plant and another inbred maize plant satisfied the written description requirement. *See Ex parte Carlson* (B.P.A.I. 2005). The Board therein stated:

> All that is required by the claims is that the hybrid has one parent that is a plant of corn variety [inbred]. Since the examiner has indicated that the seed and the plant of the corn variety [inbred] are allowable . . . there can be no doubt that the specification provides and adequate written description of this corn variety. In addition, the examiner appears to recognize (Answer, page 25) that appellant's specification describes an exemplary hybrid

> wherein one parent was a plant of the corn variety [inbred]. . . Accordingly, it is unclear to this merits panel what additional description is necessary.

Ex parte Carlson, p. 16. Here, the Examiner has indicated that claim 11, directed towards a plant having all the morphological and physiological traits of PH581 wherein PH581 was deposited with the ATCC, is allowable. Accordingly, the genus of hybrid plants and seeds encompassed by claims 1-10, 13-16 and 18-30 are as well.

Applicants reiterate that each member of the genus of hybrids which has PH581 as a parent and which is encompassed by claims 1-10, 13-16 and 18-30 shares the identifying structural feature of the cells and/or chromosomes of inbred line PH581. An Applicants claims are described where they set forth and define "structural features commonly possessed by members of the genus that distinguish them from others." *Regents of University of California*, 119 F.3d at 1568, 43 U.S.P.Q.2d at 1406 (emphasis added). One of skill in the art, utilizing technology well known in the art, could identify any member of the claimed genus. This is sufficient to meet the written description requirement. *See Id.* at 1568, 1406 (stating that with a fully described a genus, one skilled in the art can "visualize or recognize the identity of the members of the genus.")

Further, Applicants reiterate that the specification contains examples of the hybrids produced by PH581 in the application as filed. (Specification, pp. 40-42, Tables 3A-3C; pp. 43-49, Tables 4A-4D). A representative number of hybrids produced by utilizing PH581 as one of the inbred parents have accordingly been described. *See Regents of University of California*, 119 F.3d at 1569, 43 U.S.P.Q.2d at 1406 (stating that an Applicant "[is] not required to disclose every species encompassed by their claims even in an unpredictable art").

The Examiner states that new claims 19-21 and 19-24 are rejected because "they are drawn to plant comprising a multitude of uncharacterized single locus conversions or transgenes" and "because they are directed to plants which comprise a finite and completely characterized genome and which exhibit a finite set of traits, and which simultaneously comprise additional genes conferring additional traits". *See* Office Action, p. 4.

Applicants respectfully traverse this rejection. The relevant claimed subject matter in claims 19-24 is the plant of claim 11 comprising a transgene or gene conversion. The Examiner has indicated that claim 11 is allowable. The specification teaches multiple ways of introgressing or transforming a maize plant with various genes which encode specific protein

products which confer advantageous traits desired in the plant. (*See* Specification, p. 29, l. 22-p. 34, l. 5). The specification also teaches multiple transgenes that could be inserted into the plant of claim 11. (*See* Specification, p. 28, l. 29-p. 36, l. 4). Applicants further note that the claims are specifically drawn to a single gene conversion, and that phenotypes resulting from multigenic interactions are not the subject matter of these claims. For example, numerous exemplary transgenes for improved nutritional quality are taught on page 33 of the specification. There are many examples of single gene conversions which effect nutritional quality, see for example, as taught in the specification transforming a plant with an antisense gene of stearoyl-ACP desaturase to increase stearic acid content of the plant (page 33, lines 18-20), introduction of a phytase-encoding gene that would enhance breakdown of phytate, adding more free phosphate to the transformed plant (page 33, lines 22-25). In addition, see U.S. Patent No. 5,936,145, issued August 10, 1999, which is prior to the filing date of the instant application. Claim 39 reads as follows: "[t]he single gene conversion of the corn plant of claim 29, where the gene confers enhanced yield stability". Thus, a single gene that confers enhanced yield stability was known in the art prior to the filing date of the instant application. One of skill in the art would recognize that it is common to transform a maize plant with various genes in order to confer desired traits to the maize plant.

The Examiner further states that claims 25-30 are included "because they are drawn to methods of using uncharacterized descendants of the exemplified inbred in a multitude of outcrossing steps to uncharacterized breeding partners." *See* Office Action, p. 4

Applicants respectfully traverse this rejection. Claims 25-30 are directed towards methods for developing a maize plant in a plant breeding program where the maize plant of claim 11 is used as a source of breeding material. The language of claims 25-30 makes clear that the maize plant of claim 11 must be used as breeding material in the breeding program described by claims 25-30.

Plant breeding techniques are well known to individuals skilled in the art. The Specification describes many of these known techniques. (Specification, p. 3, l. 19-p. 7, l. 17). In particular, the specification discusses the role of an inbred maize line in a plant breeding program:

> Plant breeding techniques *known in the art and used in a maize plant breeding program* include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, restriction length polymorphism enhanced selection, genetic marker enhanced selection

> and transformation. The development of maize hybrids in a maize plant breeding program requires, in general, the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection breeding methods are used to develop inbred lines from breeding populations. Maize plant breeding programs combine the genetic backgrounds from two or more inbred lines or various other germplasm sources into breeding pools from which new inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which of those have commercial potential. (Specification, p. 3, l. 20-p.4, l. 1) (emphasis added).

As the specification makes clear, one of ordinary skill in the art would know how a maize inbred line is to be used in a plant breeding program. As taught by the specification, the maize inbred is used as a source of germplasm in creating new hybrid lines. It is thus clear from the specification, and to one of ordinary skill in the art, how PH581 would be employed in a plant breeding program.

One skilled in the art would thus recognize that Applicants were in possession of the invention described in claims 1-10, 13-16 and 18-30 as of the filing date of the application. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph.

C. Enablement regarding Claims 1-10, 13-16 and 18-30

Claims 1-10 remain and claims 13-16 and 19-30 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. The Examiner asserts that the claims(s) contains subject matter, which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention for the reasons stated in the Office Action of December 17, 2004. *See* Office Action, p. 4

Applicants respectfully traverse. Applicants reiterate that the claimed F1 hybrid seed is routinely and easily produced by crossing a plant from inbred maize line PH581 with a plant from another inbred maize line. Applicants have described how to produce an F1 hybrid from inbred maize line PH581. (Specification, p. 5, l. 1-p. 7, l. 17). Applicants have also made a deposit of inbred PH581 that fully enables others to obtain the inbred seed needed to make the claimed F1 hybrids.

Applicants have also provided a working example showing the production of an F1 hybrid produced from the cross of inbred PH581 and inbred PH6WG (*See* Tables 4A-4D, Specification, p. 43-49). The Examiner has shown no evidence as to why this working example does not show enablement of claims 1-10, 13-16, and 18-30, directed to hybrid maize plants, plant parts and seeds produced by crossing maize inbred line PH581 with another maize plant. As shown by Tables 4A-4D, PH581 demonstrates good specific combining ability with other inbreds (*See* Tables 4A-4D, Specification, p. 43-49). One of ordinary skill in the art could therefore use PH581 and another maize inbred plant to create an F1 hybrid, without undue experimentation. This is sufficient to enable claims 1-10, 13-16, and 18-29. *In re Wands*, 858 F.2d at 737.

Applicants further reiterate the arguments regarding the references cited by the Examiner as previously presented in the Amendment of April 18, 2005. Applicants assert the references relate to segregating populations of seed (Kevern), selection within the segregating populations of seed (Carlone), comparison of synthetic populations (Stuber *et al.*), and the making of all possible crosses including F2, 3-way and backcrosses (Melchinger) to produce a population of seed. In contrast, the claimed invention teaches the use of stable and genetically fixed inbred lines to produce an F1 hybrid. An F1 hybrid as claimed is not a genetically mixed population, but rather is highly homogeneous and reproducible because it is made from the highly homogeneous and reproducible inbred maize line PH581. (Specification, p. 16, lines 17-18). As stated *supra*, Applicants have provided a working example showing the production of an F1 hybrid produced from the cross of inbred PH581 and inbred PH6WG (*See* Tables 4A-4D, Specification, p. 43-49). This is sufficient to comply with the enablement requirement.

Regarding claims 19-21, the Examiner cites Murray *et al.* and states that "linkage drag is common phenomenon in corn breeding, and the equivalent of 10 backcrosses resulted in the retention of 10% of the unwanted donor parent genome, in contrast to the predicted less than 1% (see, e.g., pages 82-84)." The Examiner further states that with respect to claims 19-24, "one skilled in the art would not know how to make such plant" and "would not know how to use plants exhibiting unknown traits". With respect to claims 25-30, the Examiner states "one skilled in the art would not know how to use said plants with uncharacterized genomes and exhibiting unspecified traits." *See* Office Action, p. 5.

Applicants respectfully traverse. With respect to Murray *et al.*, the Examiner is referring to a section that is discussing six backcrosses to a specific inbred line, which is not PH581. Moreover, Murray *et al.* teaches that the very issue of linkage drag cited by the Examiner can be greatly circumvented with the use of molecular markers. Murray *et al.* states: "[t]he longstanding concept of using markers flanking a desirable gene to circumvent these problems (Bartlett and Haldane, 1935) is now practical with RFLP markers. Individuals in which recombination has occurred optimally close to the desired locus can be identified and thus linkage drag can be greatly reduced." (Murray *et al.*, p. 84).

Moreover, the specification teaches on page 4, lines 12-18, that "[b]ackcrossing can be used to transfer a specific desirable trait from one inbred or source to an inbred that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (recurrent parent) to a donor inbred (non-recurrent parent), that carries the appropriate gene(s) for the trait in question. The progeny of this cross is then mated back to the superior recurrent parent followed by selection in the resultant progeny for the desired trait to be transferred from the non-recurrent parent." Further, the specification provides a description of how to backcross traits into PH581. (Specification, p. 23, l. 33-p. 36, l. 4).

In addition, the specification provides a description of how to backcross traits into PH581 (Specification, p. 22, ll. 16-31) and it is understood by those of skill in the art that backcross conversions are routinely produced and do not represent a substantial change to a variety. The World Seed Organization, on its web site, writes, "[t]he concept of an essentially derived variety was introduced into the 1991 Act of the UPOV Convention in order to avoid plagiarism through mutation, multiple back-crossing and to fill the gap between Plant Breeder's Rights and patents." ASSINSEL, an International breeders association, has published a position paper that refers to a conversion produced by repeated backcrossing of parental lines of hybrid varieties as a "cosmetic modification". As determined by the UPOV Convention, "essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering" (emphasis added). A copy of the relevant portion of the UPOV Convention and the World Seed Organization web site was attached as Appendix F to an amendment filed March 11, 2003 in the parent case, U.S. Serial No. 09/758,802, now U.S. Patent No. 6,717,037. Thus, it is clear that there is worldwide agreement that by obtaining the seed of a

newly developed variety such as PH581, and by using such seed for repeated backcrossing in accordance with the current claims, one is producing only a cosmetic modification and plagiarizing the work of the inbred inventor.

The ability of one of ordinary skill in the art to effectively use backcrossing to introgress a single gene conversion is also clearly supported by the scientific literature. For example, see Ragot, M. *et al.* (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires (Les Colloques*, Vol. 72, pp. 45-56 (attached as Appendix 1), and Openshaw *et al.*, (1994) Marker-assisted Selection in Backcross Breeding, Analysis of Molecular Marker Data, pp. 41-43 (attached as Appendix 2). Specifically, Ragot *et al.*, which makes note of the earlier work of Murray *et al.*, demonstrates that "spectacular" progress toward the recurrent parent genotype was obtained with 61 RFLP markers. Ragot *et al.* concludes that "recovery of the recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated."

As to claim 22, the Examiner cites Goldman *et al.* and states that "the use of molecular markers to facilitate the identification of chromosomal regions associated with quantitatively inherited traits is hampered by the different linkage maps generated when different breeding lines are used as parents." *See* Office Action, p. 6.

Applicants respectfully traverse. Goldman *et al.* is discussing the identification of specific loci associated with specific traits, i.e. high and low oil content, in specific strains, specifically Illinois High Oil and Illinois Low Oil. (See, e.g., Goldman *et al.*, p. 908). It does not discuss the use of molecular markers generally to identify other traits in other lines of maize, let alone PH581.

Goldman *et al.* does not state that molecular markers can not be used in the identification of specific traits. Instead, Goldman *et al.* discusses the value of using markers in identifying various traits in a maize line. For example, Goldman *et al.* states: "[t]he recent development of molecular marker technology has enabled the association of DNA markers with important agronomic traits such as yield, plant height, and disease resistance." (Goldman *et al.*, p. 908).

Furthermore, the specification teaches multiple ways of introgressing or transforming a maize plant with various genes which encode specific protein products which confer advantageous traits desired in the plant. (Specification, p. 23, l. 33-p. 36, l. 4). This includes the

use of markers to aid in the identification, selection and transformation of the maize plant with the desired gene. (For example, *see* U.S. Patent No. 6,717,037, Table A, column 12, line 4 through column 13, line 61 which disclose the SSR markers in the present application).

Applicants have described how to produce an F1 hybrid from inbred maize line PH581. In addition, one skilled in the art of corn breeding would know that the F1 plants and seed of claims 1-10, 13-16 and 18-30 can routinely and easily be produced by crossing PH581 with another inbred maize line. Further, one skilled in the art of corn breeding would also know that the maize plants of claims 18-30 can easily be engineered to contain and express foreign genes. Accordingly, Applicants submit that claims 1-10, 13-16, and 18-30 are fully enabled and have fully satisfied the legal standards for enablement.

Rejections Under 35 U.S.C. § 102(b)

Claim 16 is rejected under 35 U.S.C. § 102(b) as being anticipated by each of Kevern (U.S. Patent No. 5,850,009) and Carlone (U.S. Patent No. 5,763,755). The Examiner states that because "the claim is drawn to seeds produced on an F1 hybrid plant . . . such progeny are indistinguishable from any known non-exemplified corn plant, including those taught by each of Kevern and Carlone". *See* Office Action, p. 7.

Applicants respectfully traverse this rejection. Neither Kevern nor Carlone disclose each of the limitations of claim 16. Claim 16 is drawn to an F2 maize seed, which is produced by growing the F1 maize plant of claim 15 and harvesting the resultant maize seed. The F1 maize plant of claim 15 is produced by from the hybrid seed of claim 14, which ultimately depends from claim 12. Claim 12 requires that the F1 hybrid seed is produced by crossing a first parent maize plant with a second parent maize plant, wherein one or both of the parent maize plants are PH581.

Neither Kevern nor Carlone teach the seed or plant of PH581, or an F1 seed or plant produced from PH581. An F2 hybrid seed produced from an F1 seed which is the result of a cross where PH581 is at least one of the parents will contain at least some of the common structural feature of PH581 which is contained in the F1 seed. Claim 11, drawn to the PH581 maize plant, has been allowed by the examiner. Therefore, because Kevern or Carlone does not teach PH581, it can not anticipate claim 16.

In light of the above, Applicants respectfully request that the Examiner reconsider and withdraw the rejections to claim 16 is under 35 U.S.C. § 102(b) as being anticipated by each of Kevern (U.S. Patent No. 5,850,009) and Carlone (U.S. Patent No. 5,763,755).

Summary

Applicants acknowledge that claims 1-15 and 17-30 are deemed free of the prior art. This clearly indicates that maize inbred line PH581 as a whole is considered to be distinguishable from the prior art for the purposes of novelty and non-obviousness.

Applicants also acknowledge that claims 11-12 and 17 are allowed.

Conclusion

In conclusion, Applicants submit in light of the above amendments and remarks, the claims as amended are in a condition for allowance, and reconsideration is respectfully requested. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

No fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Reconsideration and allowance is respectfully requested.

Respectfully submitted,

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Marker-assisted backcrossing: a practical example

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Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC_6 generation were obtained at the BC_3 generation, about one year after BC_1 seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure, if at all possible. Theoretically, a minimum

APPENDIX 1

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1989) reported about 90% recurrent parent genotype recovery in two BC_{10}-equivalent conversions (A632Rr and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tanksley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgene construct) from a donor into a recipient maize line.

Materials and methods

Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgenic construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Koziel *et al.* 1993). Transformation was achieved through microprojectile bombardment (Koziel *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

Backcross protocol

The F1 progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC_1 progeny.

For each backcross generation, except the BC_4, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC_4 plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker analyses. Results of marker an flowering. A single plant was rescued and transferred onto it embryos first underwent a gro culture medium, before being average, four months.

Molecular marker analys

Restriction Fragment Le genotypes in all four genera chemiluminescent techniques. I were chosen from among those provided coverage of the entire contained two loci tightly linke recombination units away (Figu BC_{n+1} generation comprised bo or tightly linked ones, and addi selected BC_n plant was heteroz independent reference populati generation.

Selection procedure

At each generation plants recurrent-parent-genotype and attempt to integrate both crite missing values were not include contributed to the selection proc best ranking one of those for w for the BC_3 selection) was avail

Results and discussion

Selection for the gene o

The observed segregatio significantly different ($P < 0.05$

Recurrent parent genoty

Statistics for the genoty performed taking the whole g backcross-derived plant theref

analyses. Results of marker analyses were made available at the latest two weeks after flowering. A single plant was selected, of which all backcross-derived embryos were rescued and transferred onto tissue culture medium. Plantlets that developed from these embryos first underwent a greenhouse acclimation phase, while still growing on tissue culture medium, before being transplanted into multipots. Backcross cycles lasted, on average, four months.

Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish genotypes in all four generations. RFLP detection involved either radioactive or chemiluminescent techniques. For the BC_1 generation, 61 marker-enzyme combinations were chosen from among those revealing polymorphism between donor and recipient. They provided coverage of the entire genome, defining intervals of about 25 cM in size, and contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16 recombination units away (Figure 1). For subsequent generations, markers analyzed in the BC_{n+1} generation comprised both those for which the selected BC_n plant was heterozygous, or tightly linked ones, and additional ones located in chromosomal segments for which the selected BC_n plant was heterozygous (Table 1). Marker map positions were obtained from independent reference populations and confirmed by analysis of segregation in the BC_1 generation.

Selection procedure

At each generation plants were ranked based both on the percentage of homozygous recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an attempt to integrate both criteria. Plants for which two or more adjacent markers had missing values were not included in the analyses. Success or failure of the pollinations also contributed to the selection procedure. One single plant was selected at each generation: the best ranking one of those for which a backcross progeny of size 100 or more (50 or more for the BC_3 selection) was available.

Results and discussion

Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not significantly different ($P < 0.05$) from the expected 1:1, as shown by Chi-square tests.

Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were performed taking the whole genome into account, including the *Bt* locus. The "perfect" backcross-derived plant therefore counts one heterozygous chromosome segment, that

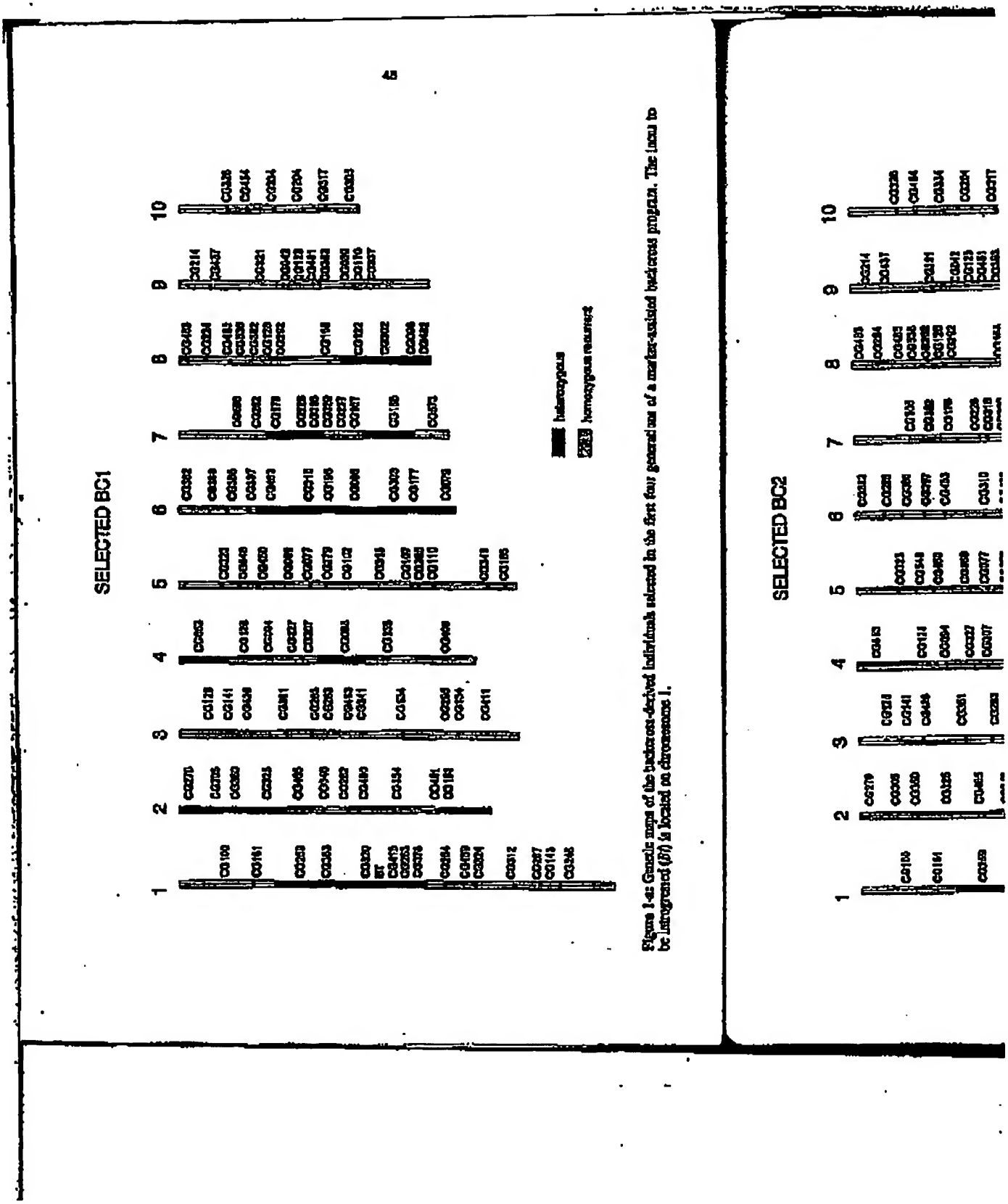

Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*bt*) is located on chromosome 1.

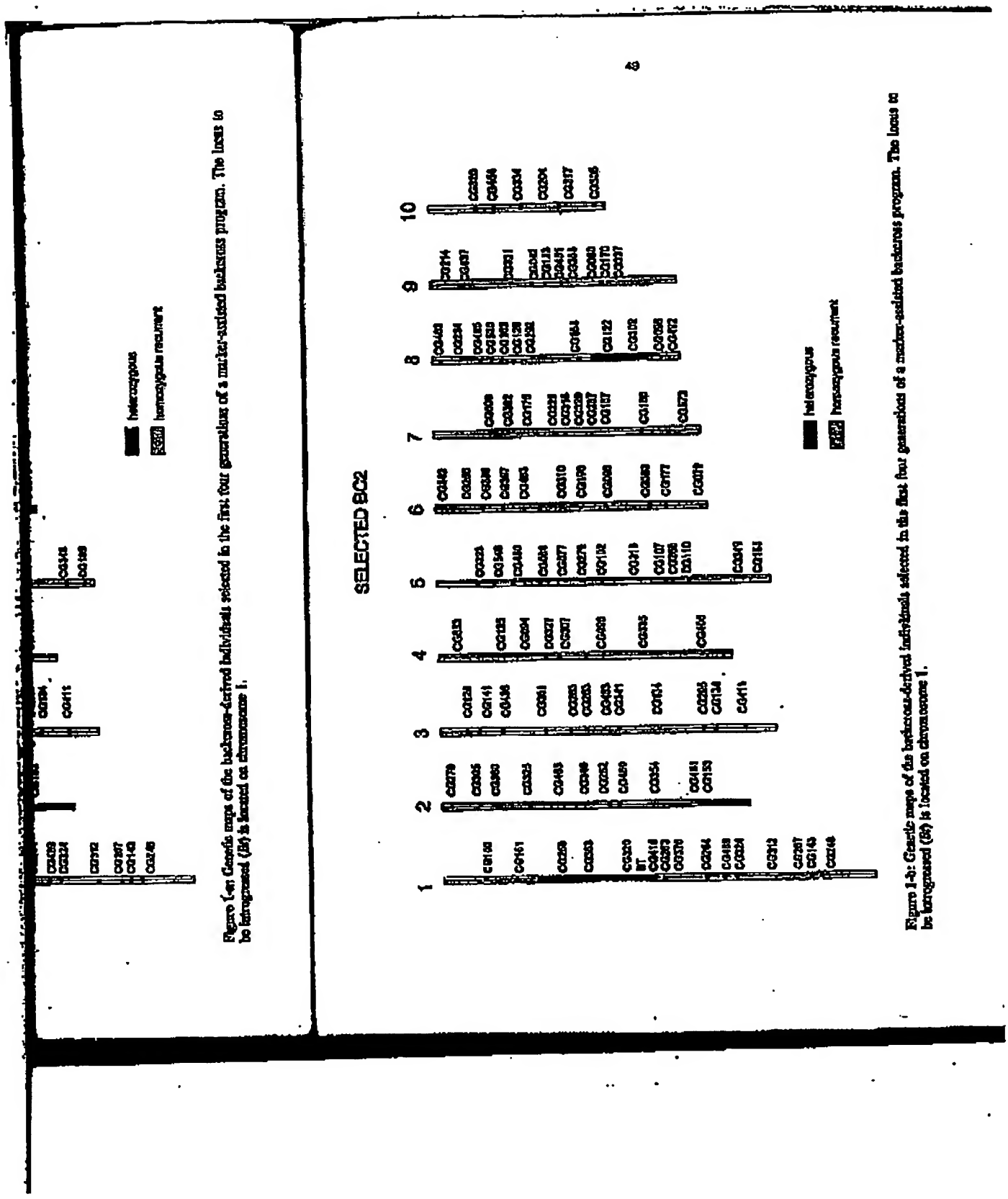

Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Sh) is located on chromosome 1.

Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Sh) is located on chromosome 1.

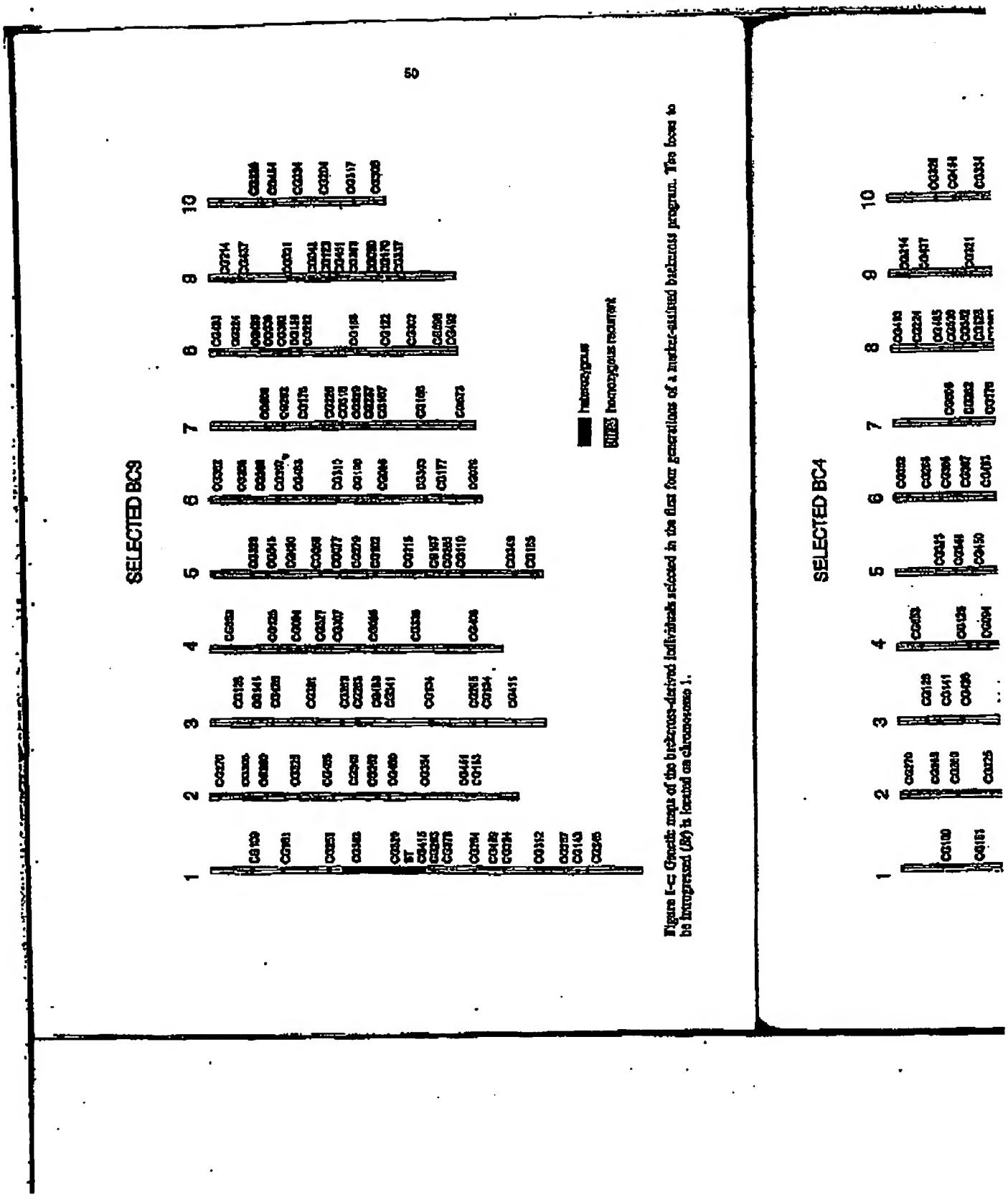

Figure 1-c: Genetic maps of the birbanus-derived individuals selected in the first four generations of a marker-assisted backcross program. The boxes to be homozygous recurrent (BR) is located on chromosome 1.

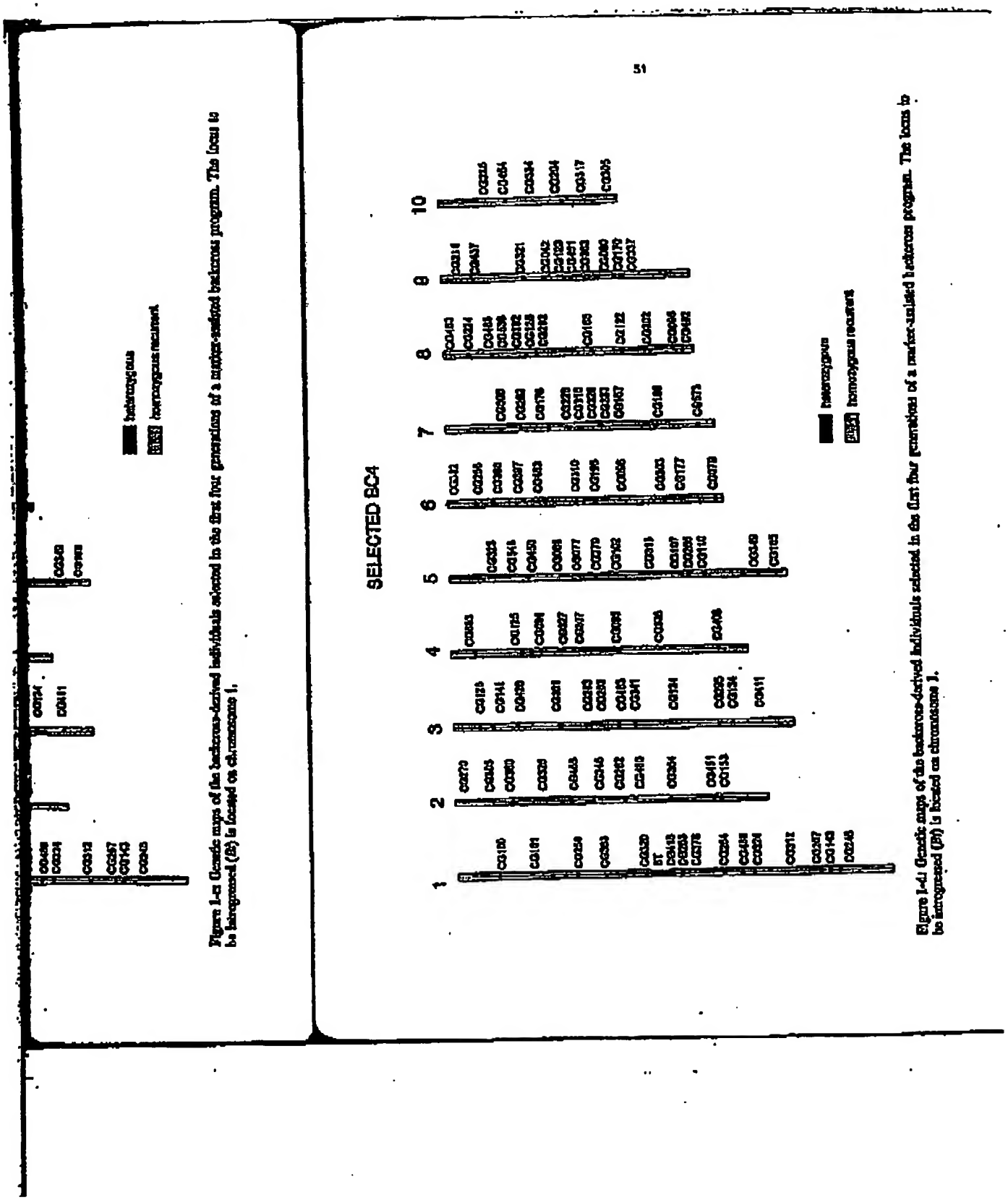

Figure 1-4a Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

Figure 1-4b Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

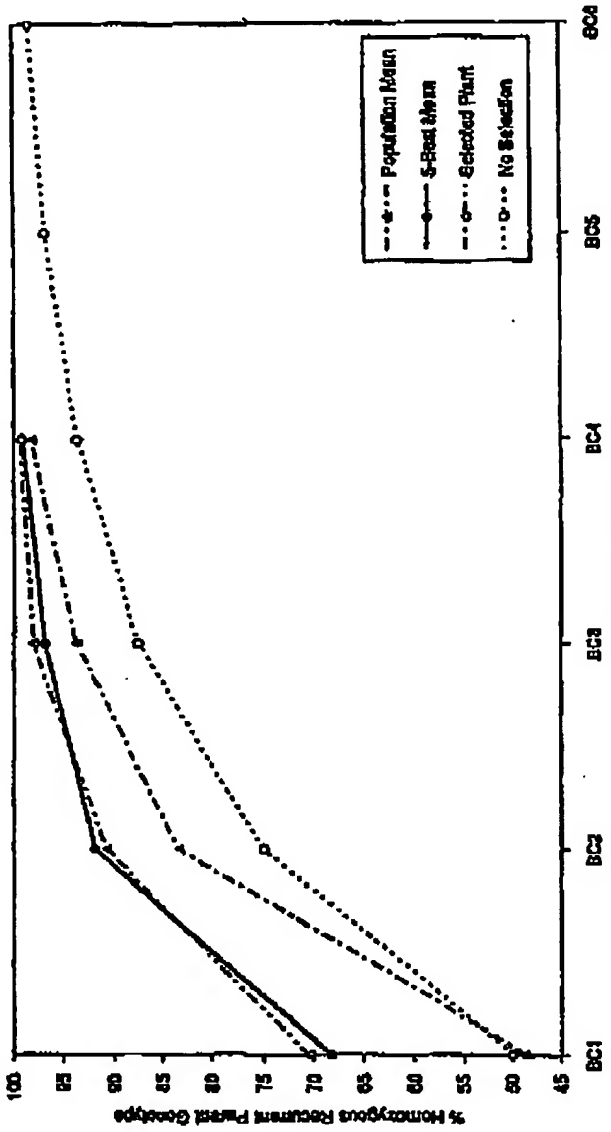

Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

	nb crossings	% chromosome	RFLP cumulated	nb plants	% homozygous recurrent	nb heterozygous	...

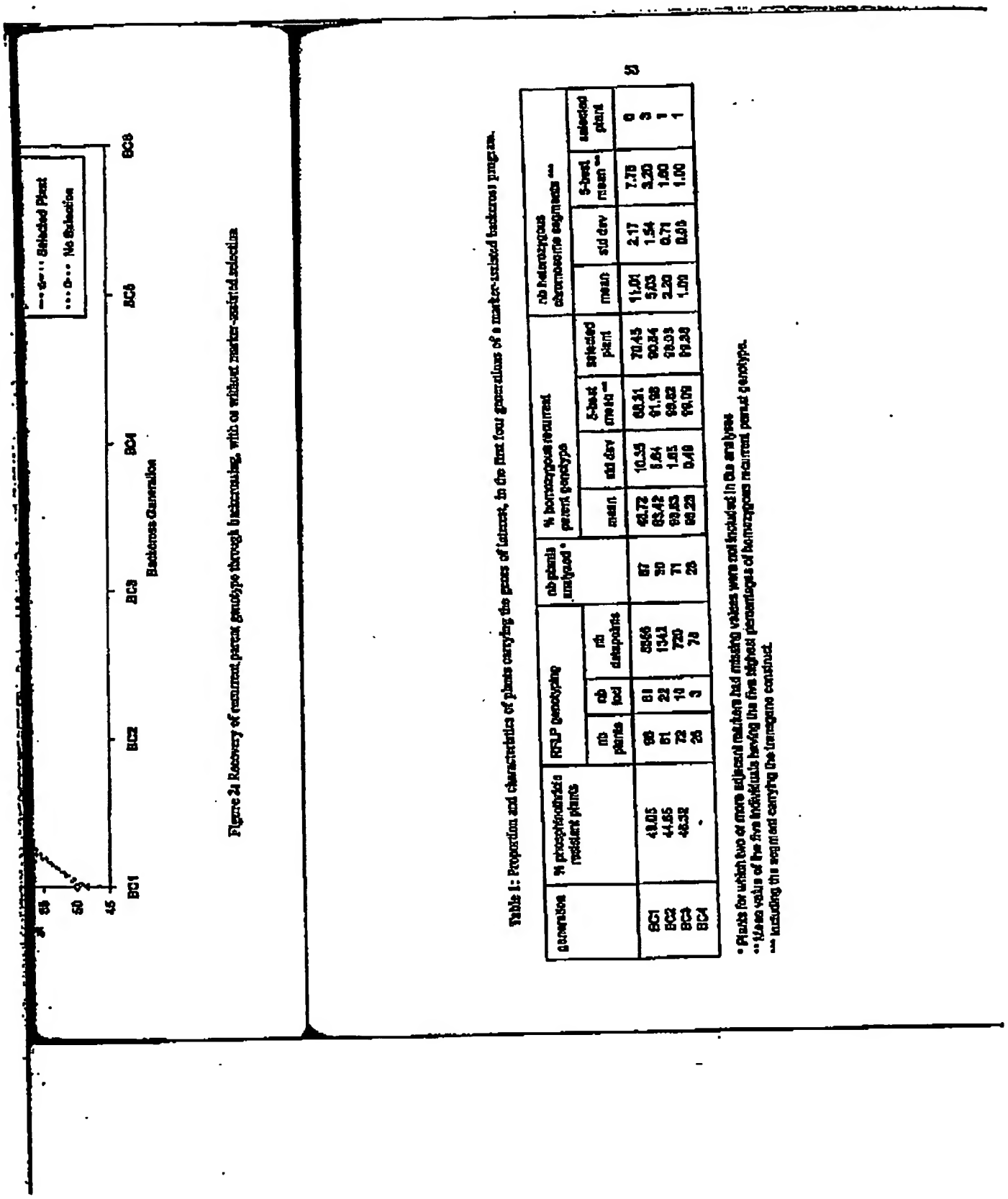

Figure 2a Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype				nb heterozygous chromosome segments **			
		nb plants	nb loci	nb datapoints		mean	std dev	5-best mean **	selected plant	mean	std dev	5-best mean **	selected plant
BC1	48.05	98	81	8566	87	92.72	10.55	98.31	70.45	11.01	2.17	7.75	9
BC2	44.65	81	22	1343	80	83.42	5.84	91.98	90.34	5.03	1.54	3.20	3
BC3	48.38	72	10	720	71	89.83	1.85	93.62	93.03	2.29	0.71	1.60	1
BC4	-	26	3	78	26	98.23	0.48	99.09	99.38	1.00	0.00	1.00	1

* Plants for which two or more adjacent markers had missing values were not included in the analyses
** Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.
*** Including the segment carrying the transgene construct.

comprising the *Br* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Br* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the BC_1 generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Br* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Br* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the BC_2 generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Br* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected BC_1 plant was almost equal to that of an unselected BC_2, that of the selected BC_2 was larger than that of an unselected BC_3, that of the selected BC_3 was barely smaller than that of an unselected BC_6, and that of the selected BC_4 was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Jarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used, BC_3 and BC_4 plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Br* locus.

Linkage drag

Linkage drag around the *Br* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected BC_1 individual, between 17.6 and 34.8% for the selected BC_2, between 2.0 and 24.0% for the selected BC_3, and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected BC_4.

The two values given for each ge correspond to extreme positions o flanking the transgene construct locu BC_4 is likely to be less than 1.5% appear to be somewhat high, reflect drag, it is much lower than what v (Stam and Zeven 1981; Tanksley *et* of tomato cultivars obtained by a b Tanksley (1989) found that the sizes cM.

Conclusion

These results clearly demonstr quality advantages over classical l through backcrossing. Only four ba than a year and a half from plant genotypically fully converted. Neve genotype could proceed even faste appropriate protocol and resources allocated.

Comparison of BC_4-derived l markers and agronomic performanc order to confirm the completeness o

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14.5 cM) for the selected BC_4.

The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC_4 is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeven 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm-2* conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC_1's, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC_4-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

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Marker-assisted Selection in Backcross Breeding

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Abstract. The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Rinke and Sears, 1961; Shaver, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The F_1 is crossed back to the RP to produce the BC_1 generation. In the BC_1 and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100\,[1-(0.5)^{n+1}]$$

where n is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for %RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. *Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.*

Generation	%RP
F_1	50.0000
BC_1	75.0000
BC_2	87.5000
BC_3	93.7500
BC_4	96.8750
BC_5	98.4375
BC_6	99.2188
BC_7	99.6094

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APPENDIX 2

Materials and methods

The maize genome was the model for the simulation. The simulated genome contained ten 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ($\lambda = 2$) (Haenson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.
Backcross generations: BC_1, BC_2, and BC_3.
Number of markers: 20, 40, 80, or 100.
Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP). %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an inbred is reduced from about seven to three.

By the BC_2 generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly become non-informative; i.e., selection causes the marker loci to become fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50 BC_1 plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best BC_2 recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10 BC_2 plants from each selected BC_2, the best BC_3 recovery had an estimated %RP of 94.6%.

Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the BC_1. However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. Percent recurrent parent genome during marker-assisted backcrossing.

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_1	84.5	84.5	84.2	88.0	89.9	90.7	90.2	90.5
BC_2	95.0	95.2	95.1	97.2	96.5	97.7	98.5	98.6
BC_3	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
				Five selected				
BC_1	82.9	85.1	84.9	84.7	87.7	88.1	88.9	88.9
BC_2	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
BC_3	97.1	98.3	98.6	98.9	97.3	98.5	99.3	99.3

Table 3. Estimates of percent recurrent parent genome, based on marker loci.

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_1	98.7	97.8	95.6	97.2	100.0	99.1	98.6	98.0
BC_2	100.0	99.8	99.3	99.5	100.0	100.0	99.9	98.2
				Five selected				
BC_1	96.4	96.5	96.2	95.8	100.0	98.5	98.3	98.2
BC_2	99.9	99.8	99.3	99.1	100.0	100.0	99.9	99.8

part of a marker-assisted backcross program. Ideally, the markers used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the results (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 28 cM in the BC_1, BC_2, and BC_3 generations, respectively (Hanson, 1959; Naveira and Barbadilla, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively and use by the breeder once the data is adequately summarized; however, Hospital et al. suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

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